Dopamine binds a D1-D2 heteromer coupled to G_q to activate a phospholipase C dependent mechanism to increase dendritic branching in the developing Medium Spiny Neuron

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Abstract

The Medium Spiny Neuron (MSN) composes approximately 95% of the neurons in the striatum in the brain. MSNs are GABAergic neurons that modulate the movement and reward pathways. Cortical and substantia nigra pars compacta neurons release glutamate and dopamine on MSNs, respectively. These inputs are required for the MSN to grow into its typical highly branched, spiny morphology. The Lanier lab found that dopamine increases dendritic branching in the developing MSN. The goal of the current study is to find the mechanism by which dopamine enhances MSN dendritic arborization. The hypothesis is that dopamine increases dendritic branching by binding a D1-D2 heteromer coupled to G_q, which a activates phospholipase C (PLC) dependent mechanism. A striatal-cortical co-culture prepared from day 16 mouse embryos was used to grow MSNs with their afferent cortical neurons. The experimental treatments were: 1) D1 receptor agonist SKF81297, D2 receptor agonist quinpirole, and both SKF81297 & quinpirole, 2) chemogenetic activation of G_q, and 3) PLC antagonist U73122. Treatments were administered *in vitro* at day 4, and regularly administered until fixation at day 19. It was found that SKF81297 and quinpirole, together and in isolation, were not able to replicate dopamine's increased branching effect. In addition, it was found that G_q activation, using a chemogenetic approach, mimicked dopamine's effect. Further, U73122 had no effect on branching on its own, but U73122 significantly attenuated dopamine's branching effects. Taken together, this data support the hypothesis that dopamine enhances branching by binding a D1-D2 heteromer coupled to G_q, to activate a PLC-dependent mechanism.

Key Terms: Medium Spiny Neuron, dopamine, developing, dendritic arborization, D1-D2 heteromer, G_q, phospholipase C, striatal-cortical co-culture

Contents

1 Introduction

1.1 Background

A neuron is a cell that forms treelike branches called dendrites, which allow it to receive information in the form of neurotransmitters. In a mature neuron, axon release of neurotransmitter into a synapse results in a well characterized response, where a neurotransmitter diffuses across the synapse and binds receptor on the receiving (post synaptic) neuron's dendrite. However, in the developing brain, neurons express plasma membrane receptors for neurotransmitters before they even form synapses (Burden and Scheiffele, 2013). Nearby neural tissue synthesizes and releases neurotransmitters, which diffuse through the interstitium, bind local cells, and influence their development (Herlenius and Lagercrantz, 2004). Without exposure to neurotransmitters during this critical period in development, a neuron will not grow into its expected morphology, which can result in neurodevelopmental disorders such as autism (Money and Stanwood, 2013). Our previous work found that the neurotransmitter dopamine increases the dendritic branching of a developing Medium Spiny Neuron (MSN) (Penrod et al., 2015). The objective of the current study is to find the mechanism dopamine initiates to increase branching. Our hypothesis is that dopamine binds a D1-D2 heteromer coupled to G_q to activate a phospholipase C (PLC) dependent mechanism.

MSNs are medium-sized and possess highly branched dendrites with a spherical dendritic arbor, meaning the dendrites radiate out from the soma equally in every direction. These dendrites are covered in tiny spines, hence the name Medium *Spiny* Neuron. Forming an extensive dendritic arbor and growing dendritic spines are crucial events that must occur during MSN development for normal brain function (Klapstein et al., 2001; Nishijima et al. 2014). MSNs compose approximately 95% of the neurons of the striatum, a brain structure that plays an

important role in movement, cognitive functions, and emotional-motivational behavior (Yager et al., 2015; Langen et al., 2011). The striatum is divided into the ventral striatum and the dorsal striatum. The ventral striatum contains the nucleus accumbens and the dorsal striatum contains the caudate and putamen. Both parts of the striatum receive input from almost all areas of cortex and the substantia nigra pars compacta. Cortical neurons release glutamate on MSNs, while the substantia nigra pars compacta neurons release dopamine. In turn, MSNs release GABA, an inhibitory neurotransmitter, onto other regions of the basal ganglia, a group of brain structures involved with movement and the reward pathways (Lanciego et al., 2012). For example, when a neuron in the motor cortex is activated, it will release the excitatory neurotransmitter glutamate onto an MSN, which will inhibit downstream basal ganglia neurons, enabling a person's voluntary movements to be smooth.

The level of dendritic branching in neurons is highly developmentally regulated. Early in brain development, neurons rapidly form an extensive, highly branched dendritic arbor, and thus establish many synaptic contacts. Postnatally, dendrites are slowly pruned over time, which is crucial for enabling the neuron to integrate and properly respond to all of the exogenous input it receives. In the human cortex, the dendritic pruning process begins at 3 months postnatal and continues until approximately 16 years old, during which time 40% of cortical synapses are lost (Karlsgodt et al., 2008). However, an overactive pruning process that too drastically reduces dendritic branching is thought to result in schizophrenia (McGlashan, 2000). To avoid disease phenotypes, neurons must have appropriate levels of dendritic branching so the right amount of synapses are present at every developmental stage (Kalsgodt et al., 2008).

Fully developed neurons must also maintain sufficient dendritic branching for normal function. Mature MSNs with stunted dendrites and a reduced spine density are implicated in

neurodegenerative diseases such as dementia with Lewy bodies, Huntington's disease, and Parkinson's disease (Zaja-Milatovic et al., 2006; Klapstein et al., 2001; Deutch, Colbran, and Winder, 2006). Huntington's disease (HD), also called "the disease of the striatum", provides the most direct example (Ehrlich 2012). HD is a genetic disorder characterized by the expansion of a CAG repeat coding for the huntingtin protein. Too many repeats encodes for mutated huntingtin protein that accumulates in neurons, forming aggregates that interfere with normal cell function and eventually cause neuronal death. MSNs are the first cell type to die in HD (Han et al., 2010). However, carriers of the HD mutation show behavioral symptoms far before striatal neurons atrophy or any other clear neurological symptoms can be detected (Lerner et al., 2012). The remaining MSNs in post-mortem HD brains show a reduced dendritic arbor and low spine density. It is believed that HD neurodegeneration begins with these morphological changes (Baquet, Gorski, and Jones 2004), which are associated with loss of MSN function and a decreased ability to generate action potentials (Lerner et al., 2012; Klapstein et al., 2001). Early behavioral HD symptoms such as apathy and depression are attributed to MSN dysfunction, while MSN death contributes to jerky, uncontrolled tremors (Miller et al., 2008; Han et al., 2010). Another example of the importance of MSN morphology occurs when afferent projections into the striatum are lost. In Parkinson's disease (PD), dopaminergic substantia nigra neurons atrophy, which results in reduced dopamine release on MSNs. MSNs respond to the absence of signal by decreasing their dendritic length and spine density (Deutch, Colbran, and Winder, 2006). This leads to impaired MSN function, contributing to the lack of motor control seen in PD patients (Deutch, Colbran, and Winder, 2006).

Many neurological, psychiatric, and mood disorders can be treated by targeting dopamine signaling pathways (Beaulieu and Gainetdinov, 2011; Cannon et al., 2008). The results of the

current study could hold clinical relevance for pregnant women taking dopamine receptor agonists/antagonists capable of crossing the placental barrier. The drug could alter fetal MSN branching or spine density. Deviations from typical MSN morphology and inability of MSNs to transmit information are highly correlated events (Klapstein et al., 2001). Thus, MSNs cannot integrate all of the cortical input they receive, resulting in an impaired striatal-cortical pathway. Impaired striatal-cortical circuits are implicated in repetitive human behaviors, including obsessive compulsive disorder (OCD) and Tourette's syndrome (Shmelkov et al., 2010).

Glutamate and dopamine are two neurotransmitters that are particularly influential in shaping MSN morphology. Contact with glutamatergic neurons is needed to induce spine formation in MSNs (Segal, Greenberger, and Korkotian 2003). Spines are the postsynaptic sites on MSNs where neurotransmitter binds receptor. Without spines, a Medium Spiny Neuron cannot perform its function of receiving and responding to glutamatergic and dopaminergic input. Until 2011, *in vitro* MSN research was conducted primarily on spineless MSNs, due to the lack of glutamatergic afferents in the culture. To produce MSNs with spines *in vitro*, our lab pioneered the striatal cortical co-culture method, which allows cortical neurons to synapse directly on striatal neurons (Penrod et al., 2011). The importance of forming synapses in the striatal-cortical co-culture cannot be understated. No-contact treatments were performed in which striatal tissue was grown in the same dish—but physically separated from—cortical tissue, which enabled MSNs to uptake secretions that cortical neurons released into the media (Penrod et al., 2015). Cortical neurons secrete glutamate, brain-derived neurotrophic factor (BDNF), and many other molecules that directly enhance MSN growth (Rauskolb et al., 2010). Our lab found that the striatal cortical co-culture had dramatically increased dendritic arborization and spine density in MSNs, in comparison to the MSNs grown in no-contact cultures (Penrod et al., 2015).

Further, the striatal-cortical co-culture is reliable and produces robust, healthy MSNs that resemble their *in vivo* counterparts on morphological and electrical measures (Penrod et al., 2011). In these striatal-cortical co-cultures, no substantia nigra tissue is present; thus, no dopaminergic neurons are present. Because of this, dopamine addition can be precisely controlled. Chronic dopamine administration to the co-cultures caused a significant increase in MSN dendritic branching compared to MSNs grown in co-cultures without dopamine (Penrod et al., 2015).

Dopamine can bind and activate all dopamine receptors. The canonical mechanism involves dopamine activating a single, monomeric G protein coupled receptor (GPCR), either D1 or D2, as shown in Figure 1. The D3, D4, and D5 receptor have undetectable expression levels in the striatum (Hurley and Jenner, 2006). Canonically, dopamine binds D1 or D2, which activate G_s or G_i, which **s**timulate or **i**nhibit adenylyl cyclase, respectively (Figure 1). D1 expressing MSNs preferentially localize to the caudate, where they innervate the substantia nigra pars reticulata (Bertran-Gonzalez et al., 2008). D2 MSNs preferentially localize in the putamen to innervate the globus pallidus (Bertran-Gonzalez et al., 2008).

However, recent evidence has found populations of MSNs that co-express both D1 and D2 receptors. Reports on the prevalence of D1-D2 co-expressing MSNs has been widely variable. However, D1 and D2 co-expression is believed to be more prevalent during MSN development. When day 18–19 rat striatum was grown in monoculture for 14 – 21 days, "virtually all" of MSNs were found to co-express both D1 and D2 (Aperia et al., 2000). This is in contrast to the adult rat brain, where MSN co-expression was found to be as low as 1.9% in the dorsal striatum (Gagnon et al., 2017).

In MSNs co-expressing both D1 and D2, a novel signaling mechanism has been found to occur, as a result of the formation of the D1-D2 heteromer. This D1-D2 heteromer is coupled to G_q, which activates phospholipase C (PLC) to eventually generate an intracellular calcium signal as shown in Figure 2 (Lee et al., 2004; Hasbi, O'Dowd and George, 2011; Rico et al., 2016). This novel D1-D2 heteromer dependent mechanism has been linked to increased neuronal growth, synaptic plasticity, schizophrenia, and depression (Perreault et al., 2010; Hasbi, O'Dowd and George, 2011; Pei et al., 2010). For example, a post mortem study revealed that humans with major depressive disorder had a significantly higher proportion of D1-D2 heteromers than control subjects, and that disruption of the heteromer had an anti-depressant effect in rats (Pei et al., 2010). In terms of characterizing the D1-D2 heteromer itself, the D1-D2 heteromer was found to be already synthesized and preassembled in the Golgi apparatus, and is translocated to the plasma membrane as a heteromer (Rico et al., 2016). The D1-D2 heteromer is connected by the carboxy terminus of the D1 receptor and the third intracellular loop of the D2 receptor (Hasbi et al., 2014).

Hasbi et al. used confocal fluorescence resonance energy transfer (FRET) to visualize the D1-D2 heteromer. Striatal neurons were exposed to two fluorophore labeled antibodies, one highly specific to the D1 receptor, the other specific to the D2 receptor. If the antibodies are in close proximity (average distance of 4 – 7 nm apart), a FRET signal will be generated due to energy transfer between fluorophores (Hasbi et al., 2009). Thus, MSNs with D1-D2 heteromers on their plasma membrane can be visualized as a FRET signal. In the adult rat brain, D1-D2 heteromers were visualized *in situ*, but limited to neurons in the nucleus accumbens, which itself only had 25% of neurons co-expressing D1 and D2 (Hasbi et al., 2009). In contrast, earlier in development, Hasbi et al. found that 90% of postnatal rat striatal neurons co-express D1 and D2,

which were distributed through the whole striatum (2009). They also confirmed that the D1-D2 heteromer used a G_q and PLC-dependent signal, resulting in intracellular calcium release (Figure 2). Like Hasbi et al., our lab is looking to support the hypothesis that the D1-D2 heteromer, G_q and the PLC pathway is a key mechanism that has physiological relevance in regulating the growth and dendritic branching of MSNs.

1.2 Goal

The goal of the current study is to find the mechanism by which dopamine increases branching. We hypothesize that dopamine increases branching by activating a D1-D2 heteromer coupled to G_q to activate a PLC-dependent mechanism, as shown in Figure 2. To test this, we grew striatal cortical co-cultures from embryonic day 16 mice for 19 days *in vitro* (div), with chronic drug administration. Our treatments involved adding specific activators or inhibitors of upstream proteins in Figure 2 to the striatal-cortical co-culture. The degree of dendritic branching of the resulting MSNs was then quantified using Sholl. The results indicate that dopamine stimulates the D1-D2 heteromer coupled to G_q to activate a PLC-dependent mechanism to increase dendritic branching in developing MSNs.

2 Methods

2.1 Animal Procedures

Animal procedures were performed at the University of Minnesota in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and in accordance with protocols approved by the University of Minnesota IACUC, as well as the principles outlined in the National Institute of Health *Guide for the Care and Use of Laboratory animals*.

2.2 Coverslip Preparation

The coverslip preparation was done several weeks prior to the dissection. Circular 12 mm German glass coverslips (Bellco; 1943-10012) were acid washed in 1 M HCl solution overnight at 55 °C, washed three times for 30 minutes with milli-Q water and then sequentially rinsed in 50%, 75%, and 95% solutions of ethanol, with 30 minute incubation periods at room temperature with each rinse. The coverslips were dried and maintained in an oven at 225°C.

Coverslip coating was performed the day before the dissection. Coverslips were transferred to 35 mm petri dishes, with five coverslips per one 35 mm petri dish. The coverslips were coated in a mixture containing 100 μg/ml poly-D-lysine (PDL, Sigma; p7886) and 4μg/ml laminin (Invitrogen; 23017015). Poly D-lysine solution was made by dissolving 100 mg of PDL in 0.1 M borate, pH 8.5. PDL/borate was passed through a 0.2 μm filter to remove particulate matter, aliquoted, and stored at −80°C. Laminin was thawed on ice, aliquoted and stored at −80°C. Right before coverslip coating, PDL was thawed at 32°C and laminin was thawed slowly on ice. Each coverslip was coated with 50 μL of PDL/laminin. The petri dish was then sealed with parafilm and incubated overnight at room temperature. After incubation, cells were rinsed

three times with sterile water and 2 mL of neuronal plating media was added to each 35 mm petri dish. The dishes were stored in the cell culture incubator until the cortical and striatal cells were ready to be plated.

2.3 The Dissection

A schematic of the striatal cortical dissection and plating process is shown in Figure 3. A mouse pregnant with day 16 fetuses was sacrificed by cervical dislocation. Her abdomen was sprayed with 70% ethanol, and sterilized serrated forceps and scissors were used to cut open her abdomen. The horns of the uterus were removed and placed into a sterile petri dish. All subsequent steps were performed in the hood while looking through a dissecting microscope at the fetuses. The fetuses, about 1.2 to 1.5 cm long at this developmental stage, were removed from the uterus and decapitated with serrated forceps. The heads were placed in the 10 cm petri dish with ~15 mL calcium and magnesium free Hank's Balanced Salt Solution (CMF-HBSS) for rinsing. The heads were then transferred to one sterile paraffin dissecting dish with CMF-HBSS so the brain could be removed from the cranium. The brain isolation and all subsequent steps of plating the neurons are shown in Figure 3. The brain isolation was done using the forceps to stab through the eyes and peeling back the thin skin and skull layer with another pair of forceps, as shown in Step 1 of Figure 3. The brain was then transferred to the second paraffin dissecting dish. The diencephalon and brain stem were discarded, leaving the cerebral hemispheres (Step 2). The cerebral hemispheres were isolated from all the mouse embryos at once. The meninges were removed from the cerebral hemispheres (Step 3). The angled forceps were used to scoop out the striatum from the medial side of the cerebral hemisphere (Step 4a). The striatum was placed in the 60 mm petri dish with 5 – 10 mL CMF-HBSS. The first third of the rostral end of

the cortex was cut out using the angled forceps (Step 4b). The cortex piece was placed in a separate 60 mm petri dish with 5 – 10 mL CMF-HBSS. Both tissues in their respective petri dishes were then chopped with a sterile razor blade. The chopped striatum was sucked up with a pipette and added to a 1.5 mL microcentrifuge tube labeled "S" (Step 6). The chopped cortex was added to a separate microcentrifuge tube labeled "C" (Step 6). To both tubes, 100 μL of 10 X trypsin EDTA and 2 μL of benzonase was added (Step 7). The tube was gently inverted and incubated 30 min at 37°C, with gentle inversions every 5 to 10 minutes during incubation (Step 8). The supernatant was removed from the settled tissue at the bottom of the microcentrifuge tube. Three 5 minute room temperature CMF-HBSS rinses were performed (Step 9). The tissue was then dissociated with a fire polished Pasteur pipette, with the tip size reduced to about half of normal (Step 10). The tissue was triturated with the Pasteur pipette approximately 10 times. The cell concentration of both striatum and cortex was counted using trypan blue and a hemocytometer (Step 11). The number of live and dead cells were recorded to assess cell viability, and these cells were plated as long as the viability was ≥ 90%. That number of live cells was used to determine the volume of tissue solution to add to the petri dishes containing the acid washed coverslips and the neuronal plating media. For all low density cultures using 35 mm dishes, 150,000 cells per dish were plated in a 3:2 cortex to striatum ratio (Step 12).

2.4 Cell Culture

After adding cells from both striatum and cortex to the dishes, the dishes were gently rocked in a 2 dimensional plus configuration to spread the cells to all areas of the dish. The cells were incubated at 37°C for 2 – 4 hours to allow the cells to adhere. The neuronal plating media was removed and replaced with growth media (0.1X Neurobasal, 1X B27, 0.2 mM Glutamine)

that was conditioned 24 – 48 hours on confluent glia cultures. Glia cultures were prepared by growing cortical tissue from postnatal day 1 – 2 mice in glia plating media (Earle's Modified Engle's Medium, 10% fetal calf serum, 0.6% glucose, 0.2 mM glutamine, 1 mM sodium pyruvate, and 1x penicillin-streptomycin). Glia cultures are then grown to 90 – 100% confluency, which takes 2 – 3 weeks, at which time they can used for cell culture. (For more detail on how to make glia conditioned media, see Penrod et al., 2011, section 2.8.) At 4 div, glia conditioned media was removed and replaced with fresh, unconditioned growth media. On day 7 and 14 div, half of the media was replaced with fresh, unconditioned growth media. Each of the three experiments in this study included a dopamine ((+)DA) treatment. For the (+)DA treatment, the cell culture was supplemented with 1 μM dopamine every 3 – 4 days, beginning at 4 div. Like dopamine administration, the experimental treatment was added to the cultures beginning on 4 div and every 3 – 4 days thereafter.

2.5 DNA Electroporation

The mCherry tagged DREADD (Designer Receptor Exclusively Activated by Designer Drugs) plasmid encodes for a modified human acetylcholine receptor, only stimulated by the inert, designer drug Clozapine *N*-oxide (CNO).

Nucleofection reagents A and B and 20 μg of the G_q DREADD plasmid were combined. Separately, the striatal neurons were dissociated in plating media immediately after the tissue was harvested. The cell number and viability with Trypan blue was used to determine the neuron concentration, and 10^6 cells were transferred to a microcentrifuge tube. Neurons were gently spun in the microfuge at 1000 g for 5 minutes. The supernatant was removed, and immediately the neurons were resuspended in Nucleofection reagent/plasmid mix. The nucleofection

reagent/plasmid/neuron solution was transferred to the nucleofection cuvette. The nucleofection cuvette was placed into the nucleofector and electroporated with the program 0-005 Neuron, Mouse Hippocampal. To the cuvette, 500 µL of warmed and pH equilibrated plating media was added. The electroporated neurons were removed from the cuvette and placed in warm plating media, yielding a final volume of 2 mL.

2.6 Immunofluorescence

Coverslips were fixed with 4% paraformaldehyde/PHEM (60 mM PIPES pH .0, 25 mM K-HEPES pH 7.0, 10 mM EGTA, 2 mM $MgCl_2$)/0.12 M sucrose-buffered fixative at 37°C for 30 minutes. The coverslips were then rinsed with 1 X PBS (phosphate buffered saline) and blocked with approximately 1 mL 3% RIA grade BSA (bovine serum albumin) in PBS and incubated overnight at 4°C. After incubation, BSA was removed and the neurons were permeabilized with 1 mL of 0.2% Triton X-100 in PBS for 10 minutes at room temperature. The cells were rinsed with PBS. The cells were then blocked with 3% RIA grade BSA in 1 X PBS and incubated for 30 minutes at room temperature. After incubation, coverslips, cell side up, were transferred to a staining box with a hydrophobic surface and overlaid with primary antibody in 1% RIA BSA/PBS. The staining box was closed to reduce evaporation and the coverslips were incubated at 4°C overnight. All antibody mixtures contained polyclonal rabbit anti-Dopamine and cyclic AMP regulated phospho-protein of 32 kDa (DARPP-32; Cell signaling, cat. #2303, 1: 250), because MSNs highly express this protein. After incubation in the primary, the coverslips were rinsed in 1 X PBS for 5 min. The coverslips, cell side up, were then overlaid with secondary antibody, in a 1:100 dilution, in 1% BSA for 1 h. Secondary antibody was conjugated to either Alexa488 or Alexa594. Following secondary incubation, coverslips were rinsed in 1X PBS and

mounted on glass slides with 2.5% 1,4-Diazabicyclco-[2.2.2]Octane, 150 mM Tris pH 8.0, and 80% glycerol mountant.

2.7 Imaging

MSN were identified by their high level of expressed DARPP-32 and imaged using a 20X objective on a Zeiss Axiovert 200M microscope controlled by MicroManager Software.

2.8 Quantification of Dendritic Branching

Neurons were traced using the Simple Neurite Tracer software plugin for Fiji/ImageJ. A traced neuron in Simple Neurite Tracer included the number and length of primaries and dendritic branches. This data was used to generate Figure 4(b)-(e). A traced neuron could also be analyzed with Sholl analysis. Sholl analysis was used to quantify the level of dendritic arborization of each individual neuron. The Sholl analysis feature of Simple Neurite Tracer placed concentric rings radiating from the center of the neuron's soma. Ring number was used as the x-axis value for the Sholl analysis figures (Figure 4a, 5, and 6), where ring 1 was the most proximal to the neuron's soma, and ring 14 was the most distal from the neuron's soma. Ring 1 had a radius equal to 10.7 μm, and each subsequent ring had a ring radii equal to $10.7n$ μm, where n is the ring number. Every time a dendrite crossed a ring loci, that counted as one crossing. The total number of crossings was the y-axis value. For example, the prototypical neuron in the dopamine treatment had a maximum number of dendritic branches (~13 crossings) at ring number 5 – 6 (Figure 4a).

Replications from 2 – 3 independent cultures were pooled per experiment. Crossings were compared at each ring number and between experimental treatments using a two-way

ANOVA with Bonferonni's post-test to determine significant differences where $p \leq 0.05$ was significant.

3 Results

The Lanier lab has found that dopamine increases dendritic branching in the developing MSN (Penrod et al. 2015). The goal of the current study is to find the mechanism by which dopamine accomplishes this. Three experiments were performed. In each experiment, we quantified dendritic branching by neurite tracing and Sholl analysis. All three experiments confirmed the previous finding that dopamine addition to the cultures resulted in increased dendritic branching from ~30 μm to ~90 μm from the soma.

3.1 D1 and D2 Receptor Agonists Fail to Replicate Dopamine's Branching Effects

The goal of the first experiment was to find the receptor(s) dopamine binds to increase branching. To do this, we selectively activated dopamine receptors by administering dopamine receptor agonists to the cultures at the same frequency dopamine was administered. We used three treatments: SKF81297, quinpirole, and SKF81297 & quinpirole. SKF81297 is a D1 receptor agonist and would activate the G_s signaling cascade shown on the left in Figure 1, as in the D1 MSN. Quinpirole is a D2 receptor agonist, and would activate the G_i signaling cascade shown on the right in Figure 1, as in the D2 MSN. SKF81297 & quinpirole would activate D1 receptors (G_s), D2 receptors (G_i), and the D1-D2 heteromer (G_q) (Lee et al., 2004).

The three agonist treatments were administered to MSNs and their resulting degree of dendritic branching was quantified using Sholl (Figure 4a). This experiment was done under the assumption that each long-term treatment would result in long-term activation of its associated pathway(s). As expected, (+)DA caused significantly more dendritic branching at rings 4 – 9, compared to (–)DA ($p \leq 0.05$) (Figure 4a). Quinpirole had no effect on dendritic branching, with similar mean number of crossings as (–)DA at all ring loci (Figure 4a). SKF81297 and

SKF81297 & quinpirole appeared to cause an increase in proximal dendritic branching and a decrease in distal branching ($p \leq 0.05$) (Figure 4a). Specifically, compared to (–)DA, SKF81297 containing treatments had increased dendritic branching at ring 2 and ring 3, while dopamine does not increase branching until ring 4 ($p \leq 0.05$) (Figure 4a). The SKF81297 & quinpirole treatment reached its maximum number of crossings at ring 4, being significantly more branched than (–)DA at this position ($p \leq 0.0001$). Both SKF81297 containing treatments have fewer mean crossings than (–)DA by ring 8, and become significantly less branched at ring 10 ($p \leq 0.05$) (Figure 4a). These results indicate that none of the treatments replicated the effects of dopamine.

The Sholl analysis provides information on the relative amount of branching between treatments, but cannot provide information on the number and length of primaries and dendrite branches. Analysis of the tracing data revealed that no treatment significantly changed the number of primaries (Figure 4b). Dopamine did increase the length of the primaries ($p \leq 0.01$) (Figure 4b). In contrast, SKF81297 reduced the length of the primary neurites in comparison to (–)DA ($p \leq 0.05$). Quinpirole and SKF81297 & quinpirole had no effect on the primary neurite length (Figure 4c). Dopamine addition caused an increase in the total number of dendritic branches off of primaries, including secondary, tertiary, and quaternary branches ($p \leq 0.01$) (Figure 4d). SKF81297 & quinpirole also caused an increase in the total number of branches, though not to the same extent as dopamine ($p \leq 0.05$) (Figure 4d). Neither SKF81297 nor quinpirole alone had an impact on the number of branches (Figure 4d). When the length of all the dendritic branches was summed, it was found that dopamine addition increased the length of dendritic branches ($p \leq 0.001$) (Figure 4e). None of the agonist treatments had any effect on the total sum length of branches compared to treatment without dopamine ((–)DA) (Figure 4e).

3.2 G_q activation using a Chemogenetic Approach

Analysis of the results in Figure 4 led us to hypothesize that dopamine acted through a different pathway from the canonical mechanism (Figure 1). The scientific literature suggests that dopamine can also act through the mechanism shown in Figure 2, in which activation of G_q is an essential upstream step. Although long-term SKF81297 and quinpirole treatment did not replicate dopamine, we believe this may be due to differences in receptor recycling induced by dopamine compared to SKF81297 & quinpirole (see discussion). Therefore, to directly determine if G_q activation was sufficient to replicate dopamine's effects, G_q was activated by administering Clozapine *N*-oxide (CNO) to mCherry tagged, DREADDs expressing MSNs (Figure 5). Prior to coverslip plating, striatal tissue was electroporated with a plasmid that encodes a designer receptor (DREADDs) that couples to the endogenous G_q. Nanomolar concentrations of CNO selectively stimulate the receptor and by extension, G_q (Zhu and Roth, 2014). It was found that 25 nM and 50 nM CNO were too highly concentrated to allow transfected MSNs to survive to day 19. Transfected MSNs were not growing because G_q was being overstimulated, resulting in toxic levels of intracellular calcium release (Figure 2). Fortunately, it was found that a 12.5 nM concentration of CNO was low enough to allow for normal MSN development. Thus, co-cultures electroporated with DREADDs were treated with long-term 12.5 nM CNO administration. If G_q is the only G protein activated in dopamine's increased branching effect, there should be no significant differences in the Sholl between the dopamine treatment and G_q activation by the CNO treatment. It was found that the dopamine treatment had slightly more branched dendrites than the CNO treatment to transfected neurons, however, no significant differences between (+)DA and (+)CNO were found (except at ring 6)

(Figure 5). As expected, dopamine addition caused increased branching from ring 2 – 11 ($p \leq 0.05$). Similarly, adding CNO to G_q coupled DREADDs neurons resulted in greater branching compared to (–)DA from ring 2 – 8 ($p \leq 0.05$). These data indicate that direct activation of the G_q pathway has an effect on MSN branching similar to that of dopamine.

3.3 PLC antagonist U71322 Reduces Dopamine's Branching Effect

The goal in experiment 3 was to find if the activator enzyme phospholipase C (PLC) is required for dopamine to increase dendritic arborization, as in the novel mechanism (Figure 2). If PLC activation is necessary to increase branching, blocking PLC will significantly reduce dopamine's branching effect. U73122 is a PLC antagonist with DMSO as the vehicle. MSNs not treated with U73122 were treated with DMSO to ensure the vehicle was in no way responsible for changing MSN dendritic branching. As expected, DMSO(+)DA had a significantly increased dendritic arbor from DMSO(–)DA ($p \leq 0.0001$) from ring 3 – 7, and ring 8 ($p \leq 0.05$) (Figure 6).

U73122 alone (U73122(–)DA) was administered to the cultures to see if blocking phospholipase C would decrease branching from basal DMSO(–)DA levels. Blocking PLC did not significantly alter branching from basal levels. This finding means that any differences in branching between DMSO(+)DA and U73122(+)DA is a result of a block in dopamine's pathway to increase branching. It was indeed found that adding U73122 significantly decreased dopamine's branching effect at rings 3 – 7 ($p \leq 0.05$). This means that dopamine's increase in branching is PLC-dependent.

U73122(+)DA was also compared to U73122(–)DA, to see if dopamine could increase branching without PLC activation. If dopamine's branching effect is PLC-dependent and

U73122 completely blocks PLC, we would expect that U73122(+)DA would not have any significant differences from U73122(–)DA. This is indeed supported by Figure 6 for most ring loci. The only locations where there is a significant difference between U73122(+)DA and U73122(–)DA is at intermittently spaced rings 3, 6, and 8 ($p \leq 0.05$). This means that the increase in branching by adding dopamine (DMSO(+)DA vs. DMSO(–)DA) is significantly greater than the increase in branching by adding dopamine in the presence of a PLC inhibitor (U73122(+)DA vs. U73122(–)DA). These data show that dopamine increases dendritic branching by a PLC-dependent mechanism.

4 Discussion

We have previously shown that dopamine increases dendritic branching in developing MSNs. Our current results demonstrate that dopamine does this by a G_q and PLC-dependent mechanism, consistent with the novel signaling pathway in which dopamine acts through a D1-D2 heteromer (Figure 2).

4.1 The Role of the D1-D2 Heteromer in Increasing Branching

The accumulation of data from Figure 4(a)-(e) was used to deduce the morphology of a prototypical neuron from each treatment (Figure 7). The dopamine treatment results in increased primary length, and increased number and length of dendritic branches. The quinpirole has no effect, across any figure. In the Sholl, both SKF81297 and SKF81297 & quinpirole appear to increase proximal branching and decrease distal branching (Figure 4a). Figure 4(b)-(e) re-affirm that SKF81297 & quinpirole cause an increase in short, proximal branches and a decrease in distal branches. Figure 4b shows that SKF81297 has short primaries which explains why dendritic branching is low distally. It is clear that the dopamine receptor agonists failed to replicate dopamine's branching effect (Figure 7).

There are several possible reasons why dopamine receptor agonists fail to replicate dopamine, including: (1) SKF81297 and quinpirole fail to activate their respective receptor, (2) The long duration of agonist application could lead to unexpected effects on receptor function and (3) There are other dopamine signaling pathways besides G_s and G_i.

SKF81297 was determined to have a high efficacy in activating adenylyl cyclase, in both *in vitro* and *in vivo* systems (Andersen and Jansen, 1990). It is well established that SKF81297 is a selective, full agonist of the D1 receptor (Andersen and Jansen, 1990). Quinpirole is a

selective, full agonist of the D2 receptor (Eilam and Szechtman, 1989). Further, both SKF81297 and quinpirole altered MSN arborization, though not in the same way as dopamine, indicating that the agonists were active. Thus, it is very unlikely that SKF81297 and quinpirole failed to activate their respective dopamine receptors.

A major difference between our study and previous studies that used SKF81297 and quinpirole to mimic the effects of dopamine is the duration of agonist exposure. In our lab, the co-culture was exposed to their respective treatment for 15 days. In contrast, previous studies measured intracellular changes from treatment addition on the order of seconds, minutes, or at a maximum of three hours after administration (Tang and Bezprozvanny, 2004; Bartlett et al., 2005). Lee et al. found that in MSNs co-expressing both D1 and D2 receptors, adding both SKF81297 & quinpirole for 20 minutes induced a PLC-dependent calcium signal similar to that induced by dopamine (2004). In an apparent contrast, our lab found that SKF81297 & quinpirole did not mimic the effect of dopamine on branching (Figure 4a). Comparison of the data from So et al. and Bartlett et al. help resolve this discrepancy (2005).

So et al. used human embryonic kidney cell line (HEK293) to study the internalization of dopamine receptors in cells co-expressing both D1 and D2 receptors (2005). Prolonged stimulation of either D1 or D2 receptor triggers invagination around the dopamine-receptor complex, which will pinch off from the plasma membrane to form an intracellular vesicle (i.e. endocytosis). After a 30 minute treatment with dopamine, or SKF81297, or quinpirole, both D1 and D2 receptors were internalized together as the D1-D2 heteromer (So et al., 2005). This means that activating one receptor can cause the D1-D2 heteromer to endocytose as one.

Bartlett et al. also used HEK293 cells, but these cells only expressed one dopamine receptor monomer, D1 or D2. After a 60 minute dopamine treatment, both the D1 and D2 receptor monomers were internalized (Bartlett et al., 2005). As an intracellular vesicle, however, the D1 and D2 receptors have different fates (Bartlett et al., 2005). The D1 receptor is quickly recycled back to the surface of the plasma membrane. Meanwhile, the D2 receptor is degraded as a result of interacting with a GPCR associated signaling protein (GASP) (Bartlett et al., 2005). Thus, new D2 receptors have to be synthesized and translocated to the plasma membrane before D2 receptor signaling can occur again. The net result is downregulation of D2 receptor signaling.

Taking these separate studies into context, our dopamine receptor Sholl results (Figure 4a) can be explained as follows. When the neurons are treated with SKF81297, the D1 receptor endocytoses and is quickly recycled to the surface repeatedly over time, causing a prolonged G_s and adenylyl cyclase dependent signal (Bartlett et al., 2005). When the cultures are treated with quinpirole, quinpirole bound to the D2 receptor endocytoses and GASP mediated degradation of the D2 receptor takes place (Bartlett et al., 2005). Over 15 days, the D2 receptor is rarely activated because D2 receptors must be continuously synthesized. This explains why no significant differences between quinpirole and (–)DA were found (Figure 7). For the SKF81297 & quinpirole treatment, both agonists bind the D1-D2 heteromer and endocytosis of the heteromer takes place (So et al., 2005). We hypothesize that quinpirole bound to the D1-D2 heteromer still allows for GASP to degrade the D2 receptor. Thus, only the D1 receptor is immediately recycled and returned to the surface of the plasma membrane, which will result in prolonged activation of G_s and adenylyl cyclase, same as SKF81297 only. This would explain the high degree of similarity in the Sholl for SKF81297 and SKF81297 & quinpirole. The ring 4 locus in SKF81297 & quinpirole that has a comparatively greater mean number of crossings than

SKF81297 can be ignored. Since it's only one ring locus, we will view that maximum value as a product of chance.

To explain why long-term treatment with dopamine has a different effect than long-term applications of SKF81297 and quinpirole, we hypothesize that these treatments trigger different recycling/degradation pathways. We propose that dopamine binds a D1-D2 heteromer. Prolonged dopamine in the environment will cause the D1-D2 heterodimer to endocytose. Upon endocytosis, we could hypothesize that GASP cannot interact with the dopamine-D1-D2 heteromer complex. In this view, dopamine, unlike SKF81297 or quinpirole, causes the D1-D2 heteromer to change conformation in a way that prevents GASP interaction. Thus, D2 is not degraded, and the heteromer returns to the surface, allowing continuous G_q signaling to occur, resulting in the significant morphological changes we see by dopamine addition (Lee et al., 2004).

Future experiments will focus on testing the hypothesis that dopamine protects the D1-D2 heterodimer from degradation, thus allowing long-term G_q signaling that leads to enhanced branching. To determine whether increased branching requires activation of the D1-D2 heteromer, a peptide that disrupts the heteromer (Hasbi et al., 2014), could be added with dopamine. If the heterodimer is essential, then this treatment should block the ability of dopamine to induce branching. A complementary test would be to add SKF83959, which has found to be a specific D1-D2 heteromer agonist and should thus mimic the effects of dopamine on branching.

4.2 The Role of G_q in Increasing Branching

Our DREADDs experiment supports the conclusion that direct activation of G_q can increase dendrite branching (Figure 5). Adding CNO was found to mimic dopamine in neurons transfected with DREADDs (Figure 5). However, Gomez et al. found that *in vivo*, 10 μM CNO can be converted to clozapine, which inhibits many receptors, including D1 and D2 (2017). Despite this, we do not believe significant amounts of CNO are being metabolized to clozapine in our cultures because (1) The increase in dendritic branching by CNO is not consistent with dopamine receptor inhibition and (2) The concentration of CNO our lab used was approximately 1000 fold less than Gomez et al.

However, to ensure that CNO being converted to clozapine is not the cause of increased dendritic branching, our DREADDs experiment will be repeated with the following controls: untransfected MSNs with and without CNO, and transfected with and without CNO. Untransfected MSNs without CNO is just the (–)DA treatment, and untransfected with CNO should look identical to (–)DA, because in the absence of the designer receptor, 12.5 nM CNO should not alter MSN growth.

To confirm the role of G_q in increasing branching, future experiments will administer both dopamine and a specific G_q inhibitor (YM 254890) to the co-culture (Takasaki et al., 2004). There will be four treatments, as the PLC inhibitor experiment, to establish that a certain concentration of G_q inhibitor alone does not affect basal dendritic branching levels.

4.3 PLC Inhibition Attenuates Dopamine's Increased Branching Effects

Inhibiting PLC in the presence of dopamine significantly reduced dopamine's ability to increase branching (Figure 6). This is the strongest evidence we have to support the hypothesis

that dopamine increases dendritic branching by a G_q and PLC-dependent mechanism. This hypothesis is supported by the finding that the PLC inhibitor alone had no impact on basal levels of dendritic growth. It was a pleasant surprise that chronically inhibiting PLC had no effect on dendritic branching or cell viability. Tang and Bezprozvanny added 10 μM PLC inhibitor U73122 for 10 minutes to demonstrate that calcium signaling is PLC-dependent (2004). We decided that a 100 fold reduction in that concentration may suffice to continuously inhibit PLC, yet still maintain cell viability long-term. It was found that 100 nM U73122 was an appropriate concentration to both effectively attenuate dopamine's effects, and not decrease branching when administered alone.

4.4 Conclusion

The goal of the current study was to find what mechanism dopamine activates to ultimately increase dendritic branching in striatal neurons. Understanding the mechanisms involved in neuronal growth can bring insight to the origins of neurodevelopmental disease, as well as disorders involving aberrant dopamine signaling, such as schizophrenia and depression.

In summary, we provide support for the theory that dopamine increases branching by binding a D1-D2 heteromer coupled to G_q, which activates a PLC-dependent mechanism. The role of the D1-D2 heteromer in this mechanism needs further support, so co-cultures will be treated with SKF83959, a specific D1-D2 heteromer agonist (Rashid et al., 2006). In addition, more evidence is needed support the hypothesis that G_q activation is required to increase dendritic branching. The possibility of CNO being converted to clozapine in our cultures must be eliminated, by employing additional controls as described. Our lab found that PLC inhibition strongly attenuated dopamine's effects on increasing dendritic branching. In conclusion, our

study supports the hypothesis that dopamine increases dendritic branching in the developing Medium Spiny Neuron by binding a D1-D2 heteromer coupled to G_q to activate a PLC-dependent mechanism.

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References

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., & Walter, P. (2002). Molecular biology of the cell. 6th Edition. New York: Garland Science, pp. (832-838)

Andersen, P. and Jansen, J. (1990). Dopamine receptor agonists: selectivity and dopamine D1 receptor efficacy. *European Journal of Pharmacology: Molecular Pharmacology*, 188(6), pp.335-347.

Aperia, A., Aizman, O., Brismar, H., Uhlén, P., Zettergren, E., Levey, A., Forssberg, H. and Greengard, P. (2000). Anatomical and physiological evidence for D_1 and D_2 dopamine receptor colocalization in neostriatal neurons. *Nature Neuroscience*, 3(3), pp.226-230.

Baquet, Z., Gorski, J., and Jones, K. (2004). Early Striatal Dendrite Deficits followed by Neuron Loss with Advanced Age in the Absence of Anterograde Cortical Brain-Derived Neurotrophic Factor. *Journal of Neuroscience*, 24(17), pp.4250-4258.

Bartlett, S., Enquist, J., Hopf, F., Lee, J., Gladher, F., Kharazia, V., Waldhoer, M., Mailliard, W., Armstrong, R., Bonci, A. and Whistler, J. (2005). Dopamine responsiveness is regulated by targeted sorting of D2 receptors. *Proceedings of the National Academy of Sciences*, 102(32), pp.11521-11526.

Beaulieu, J. and Gainetdinov, R. (2011). The Physiology, Signaling, and Pharmacology of Dopamine Receptors. *Pharmacological Reviews*, 63(1), pp.182-217.

Bertran-Gonzalez, J., Bosch, C., Maroteaux, M., Matamales, M., Herve, D., Valjent, E. and Girault, J. (2008). Opposing Patterns of Signaling Activation in Dopamine D1 and D2 Receptor-Expressing Striatal Neurons in Response to Cocaine and Haloperidol. *Journal of Neuroscience*, 28(22), pp.5671-5685.

Burden, S. and Scheiffele, P. (2013). Synapse Formation. *Fundamental Neuroscience (Fourth Edition)*, pp.385-403.

Cannon, D., Klaver, J., Peck, S., Rallis-Voak, D., Erickson, K. and Drevets, W. (2008). Dopamine Type-1 Receptor Binding in Major Depressive Disorder Assessed Using Positron Emission Tomography and [11C]NNC-112. *Neuropsychopharmacology*, 34(5), pp.1277-1287.

Deutch, A., Colbran, R. and Winder, D. (2007). Striatal plasticity and medium spiny neuron dendritic remodeling in parkinsonism. *Parkinsonism & Related Disorders*, 13, pp.S251-S258.

Ehrlich, M. (2018). *Huntington's Disease and the Striatal Medium Spiny Neuron: Cell-Autonomous and Non-Cell-Autonomous Mechanisms of Disease*.

Eilam, D. and Szechtman, H. (1989). Biphasic effect of D-2 agonist quinpirole on locomotion and movements. *European Journal of Pharmacology*, 161(2-3), pp.151-157.

Gagnon, D., Petryszyn, S., Sanchez, M., Bories, C., Beaulieu, J., De Koninck, Y., Parent, A. and Parent, M. (2017). Striatal Neurons Expressing D1 and D2 Receptors are Morphologically Distinct and Differently Affected by Dopamine Denervation in Mice. *Scientific Reports*, 7, p.41432.

Gomez, J., Bonaventura, J., Lesniak, W., Mathews, W., Sysa-Shah, P., Rodriguez, L., Ellis, R., Richie, C., Harvey, B., Dannals, R., Pomper, M., Bonci, A. and Michaelides, M. (2017). Chemogenetics revealed: DREADD occupancy and activation via converted clozapine. *Science*, 357(6350), pp.503-507.

Han, I., You, Y., Kordower, J., Brady, S. and Morfini, G. (2010). Differential vulnerability of neurons in Huntington's disease: the role of cell type-specific features. *Journal of Neurochemistry*.

Hasbi, A., Fan, T., Alijaniaram, M., Nguyen, T., Perreault, M., O'Dowd, B. and George, S. (2009). Calcium signaling cascade links dopamine D1–D2 receptor heteromer to striatal BDNF production and neuronal growth. *Proceedings of the National Academy of Sciences*, 106(50), pp.21377-21382.

Hasbi, A., O'Dowd, B. and George, S. (2011). Dopamine D1-D2 receptor heteromer signaling pathway in the brain: emerging physiological relevance. *Molecular Brain*, 4(1), p.26.

Hasbi, A., Perreault, M., Shen, M., Zhang, L., To, R., Fan, T., Nguyen, T., Ji, X., O'Dowd, B. and George, S. (2014). A peptide targeting an interaction interface disrupts the dopamine D1-D2 receptor heteromer to block signaling and function *in vitro* and *in vivo*: effective selective antagonism. *The FASEB Journal*, 28(11), pp.4806-4820.

Herlenius, E. and Lagercrantz, H. (2004). Development of neurotransmitter systems during critical periods. *Experimental Neurology*, 190, pp.8-21.

Hurley, M. and Jenner, P. (2006). What has been learnt from study of dopamine receptors in Parkinson's disease?. *Pharmacology & Therapeutics*, 111(3), pp.715-728.

Karlsgodt, K., Sun, D., Jimenez, A., Lutkenhoff, E., Willhite, R., van Erp, T. and Cannon, T. (2008). Developmental disruptions in neural connectivity in the pathophysiology of schizophrenia. *Development and Psychopathology*, 20(04), p.1297.

Klapstein, G., Fisher, R., Zanjani, H., Cepeda, C., Jokel, E., Chesselet, M. and Levine, M. (2001). Electrophysiological and Morphological Changes in Striatal Spiny Neurons in

R6/2 Huntington's Disease Transgenic Mice. *Journal of Neurophysiology*, 86(6), pp.2667-2677.

Lanciego, J., Luquin, N. and Obeso, J. (2012). Functional Neuroanatomy of the Basal Ganglia. *Cold Spring Harbor Perspectives in Medicine*, 2(12), pp.a009621-a009621.

Langen, M., Kas, M., Staal, W., van Engeland, H. and Durston, S. (2011). The neurobiology of repetitive behavior: Of mice…. *Neuroscience & Biobehavioral Reviews*, 35(3), pp.345-355.

Lee, S., So, C., Rashid, A., Varghese, G., Cheng, R., Lança, A., O'Dowd, B. and George, S. (2004). Dopamine D1 and D2 Receptor Co-activation Generates a Novel Phospholipase C-mediated Calcium Signal. *Journal of Biological Chemistry*, 279(34), pp.35671-35678.

McGlashan, T. (2000). Schizophrenia as a Disorder of Developmentally Reduced Synaptic Connectivity. *Archives of General Psychiatry*, 57(7), pp.637-648.

Miller, B., Walker, A., Shah, A., Barton, S. and Rebec, G. (2008). Dysregulated Information Processing by Medium Spiny Neurons in Striatum of Freely Behaving Mouse Models of Huntington's Disease. *Journal of Neurophysiology*, 100(4), pp.2205-2216.

Money, K. and Stanwood, G. (2013). Developmental origins of brain disorders: roles for dopamine. *Frontiers in Cellular Neuroscience*, 7.

Nishijima, H., Suzuki, S., Kon, T., Funamizu, Y., Ueno, T., Haga, R., Suzuki, C., Arai, A., Kimura, T., Suzuki, C., Meguro, R., Miki, Y., Yamada, J., Migita, K., Ichinohe, N., Ueno, S., Baba, M. and Tomiyama, M. (2014). Morphologic changes of dendritic spines of striatal neurons in the levodopa-induced dyskinesia model. *Movement Disorders*, 29(3), pp.336-343.

Pei, L., Li, S., Wang, M., Diwan, M., Anisman, H., Fletcher, P., Nobrega, J. and Liu, F. (2010). Uncoupling the dopamine D1-D2 receptor complex exerts antidepressant-like effects. *Nature Medicine*, 16(12), pp.1393-1395.

Penrod, R., Campagna, J., Panneck, T., Preese, L. and Lanier, L. (2015). The presence of cortical neurons in striatal-cortical co-cultures alters the effects of dopamine and BDNF on medium spiny neuron dendritic development. *Frontiers in Cellular Neuroscience*, 9.

Penrod, R., Kourrich, S., Kearney, E., Thomas, M. and Lanier, L. (2011). An embryonic culture system for the investigation of striatal medium spiny neuron dendritic spine development and plasticity. *Journal of Neuroscience Methods*, 200(1), pp.1-13.

Perreault, M., Hasbi, A., Alijaniaram, M., Fan, T., Varghese, G., Fletcher, P., Seeman, P., O'Dowd, B. and George, S. (2010). The Dopamine D1-D2 Receptor Heteromer Localizes in Dynorphin/Enkephalin Neurons. *Journal of Biological Chemistry*, 285(47), pp.36625-36634.

Rashid, A., So, C., Kong, M., Furtak, T., El-Ghundi, M., Cheng, R., O'Dowd, B. and George, S. (2006). D1-D2 dopamine receptor heterooligomers with unique pharmacology are coupled to rapid activation of Gq/11 in the striatum. *Proceedings of the National Academy of Sciences*, 104(2), pp.654-659.

Rauskolb, S., Zagrebelsky, M., Dreznjak, A., Deogracias, R., Matsumoto, T., Wiese, S., Erne, B., Sendtner, M., Schaeren-Wiemers, N., Korte, M. and Barde, Y. (2010). Global Deprivation of Brain-Derived Neurotrophic Factor in the CNS Reveals an Area-Specific Requirement for Dendritic Growth. *Journal of Neuroscience*, 30(5), pp.1739-1749.

Rico, A., Dopeso-Reyes, I., Martínez-Pinilla, E., Sucunza, D., Pignataro, D., Roda, E., Marín-Ramos, D., Labandeira-García, J., George, S., Franco, R. and Lanciego, J. (2016).

Neurochemical evidence supporting dopamine D1–D2 receptor heteromers in the striatum of the long-tailed macaque: changes following dopaminergic manipulation. *Brain Structure and Function*, 222(4), pp.1767-1784.

Segal, M., Greenberger, V. and Korkotian, E. (2003). Formation of dendritic spines in cultured striatal neurons depends on excitatory afferent activity. *European Journal of Neuroscience*, 17(12), pp.2573-2585.

Shmelkov, S., Hormigo, A., Jing, D., Proenca, C., Bath, K., Milde, T., Shmelkov, E., Kushner, J., Baljevic, M., Dincheva, I., Murphy, A., Valenzuela, D., Gale, N., Yancopoulos, G., Ninan, I., Lee, F. and Rafii, S. (2010). *Slitrk5 deficiency impairs corticostriatal circuitry and leads to obsessive-compulsive–like behaviors in mice*.

So, C., Varghese, G., Curley, K., Kong, M., Alijaniaram,, M., Ji, X., Nguyen, T., O'Dowd, B. and George, S. (2005). D1 and D2 Dopamine Receptors Form Heterooligomers and Co-internalize Following Selective Activation of Either Receptor. *Molecular Pharmacology*.

Takasaki, J., Saito, T., Taniguchi, M., Kawasaki, T., Moritani, Y., Hayashi, K. and Kobori, M. (2004). A Novel Gαq/11-selective Inhibitor. *Journal of Biological Chemistry*, 279(46), pp.47438-47445.

Tang, T. and Bezprozvanny, I. (2004). Dopamine Receptor-mediated Ca^{2+} Signaling in Striatal Medium Spiny Neurons. *Journal of Biological Chemistry*, 279(40), pp.42082-42094.

Yager, L., Garcia, A., Wunsch, A. and Ferguson, S. (2015). The ins and outs of the striatum: Role in drug addiction. *Neuroscience*, 301, pp.529-541.

Zaja-Milatovic, S., Keene, C., Montine, K., Leverenz, J., Tsuang, D. and Montine, T. (2006). Selective dendritic degeneration of medium spiny neurons in dementia with Lewy bodies. *Neurology*, 66(10), pp.1591-1593.

Zhu, H. and Roth, B. (2014). DREADD: A Chemogenetic GPCR Signaling Platform. *International Journal of Neuropsychopharmacology*, 18(1).

Figures

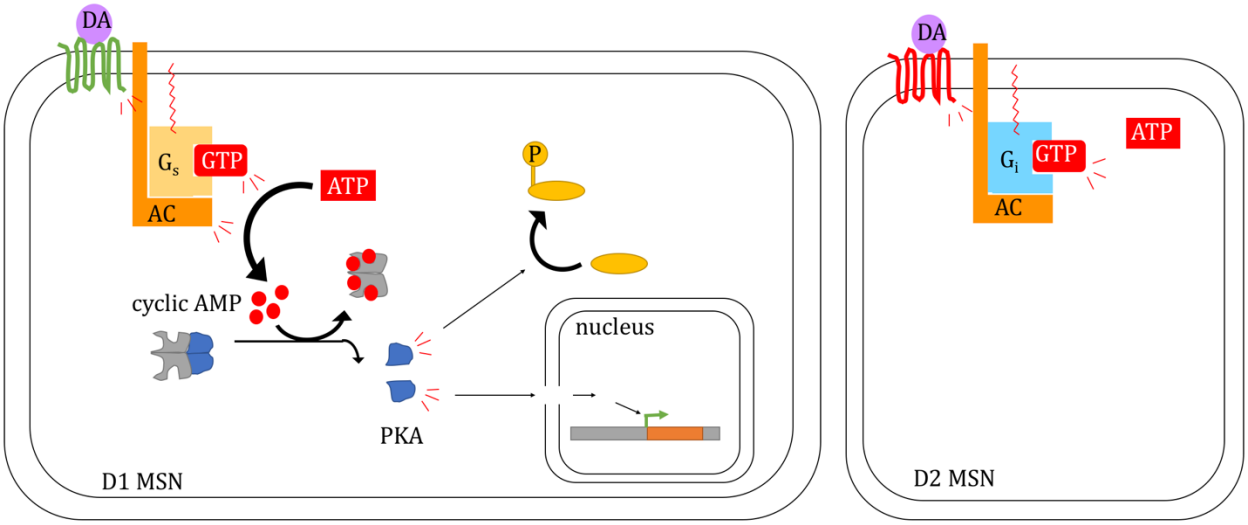

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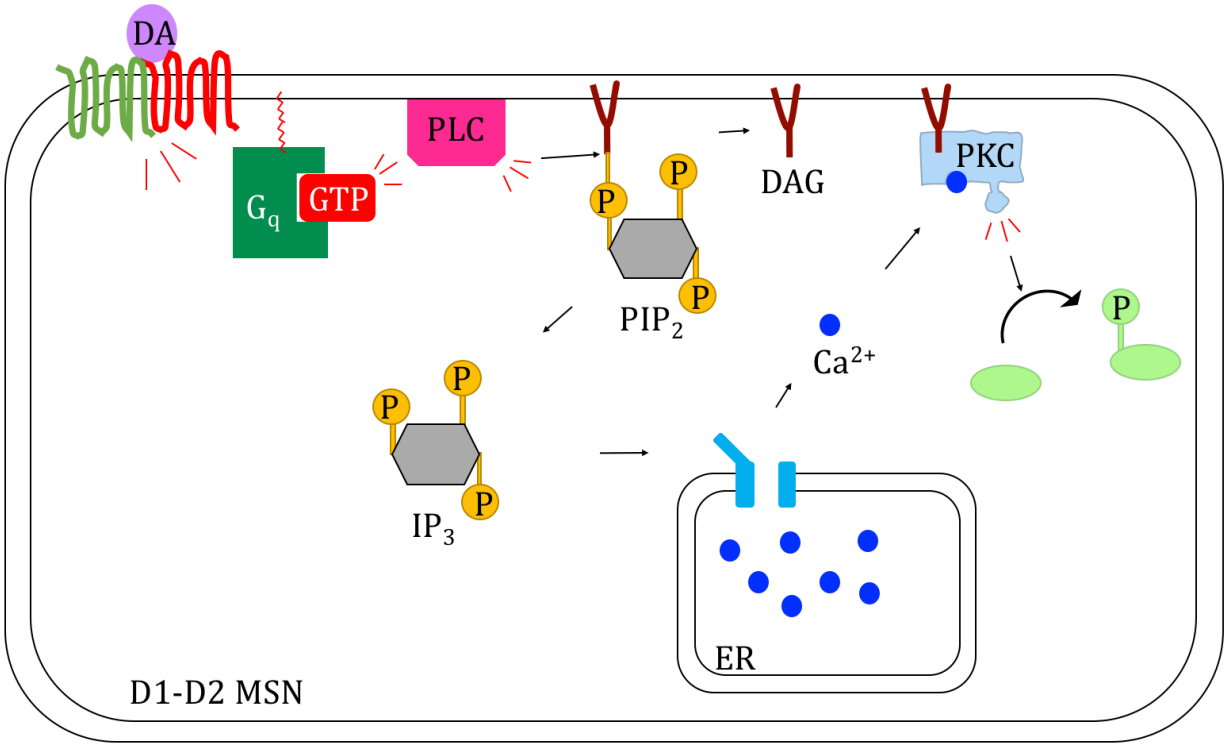
Figure 1. **The Canonical Mechanism that follows Dopamine Receptor Monomer Activation**. In this schematic, dopamine (DA) binds receptor on a MSN expressing only the D1 receptor (D1 MSN, left) or a MSN only expressing the D2 receptor (D2 MSN, right). Additionally, an agonist such as SKF81297 can bind and activate the D1 receptor (left) and quinpirole can bind and activate the D2 receptor (right). When the D1 receptor coupled to the stimulatory G protein G_s is activated, adenylyl cyclase (AC) becomes active, and catalyzes the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (AMP), which frees protein kinase A (PKA) to phosphorylate proteins and/or to enter the nucleus to alter transcription. In contrast, when the D2 receptor coupled to G_i is activated, adenylyl cyclase is inhibited, and cytosolic ATP can build up. **Note**: D1 and D2 MSNs do not differ in cell size—the D1 MSN appears larger in the schematic in order to include all of the downstream effects of cyclic AMP.



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6th edition

Figure 2: **The Novel Mechanism that follows D1-D2 Heteromer Activation.** This schematic shows the proposed mechanism by which dopamine increases dendritic branching. Dopamine (DA) binds the D1-D2 heteromer to activate G_q, which activates phospholipase C (PLC). PLC cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) to yield the products inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 causes calcium (Ca^{2+}) channels on the endoplasmic reticulum (ER) to open, resulting in intracellular calcium release. Calcium ions can activate protein kinase C (PKC) to phosphorylate proteins (green oval).

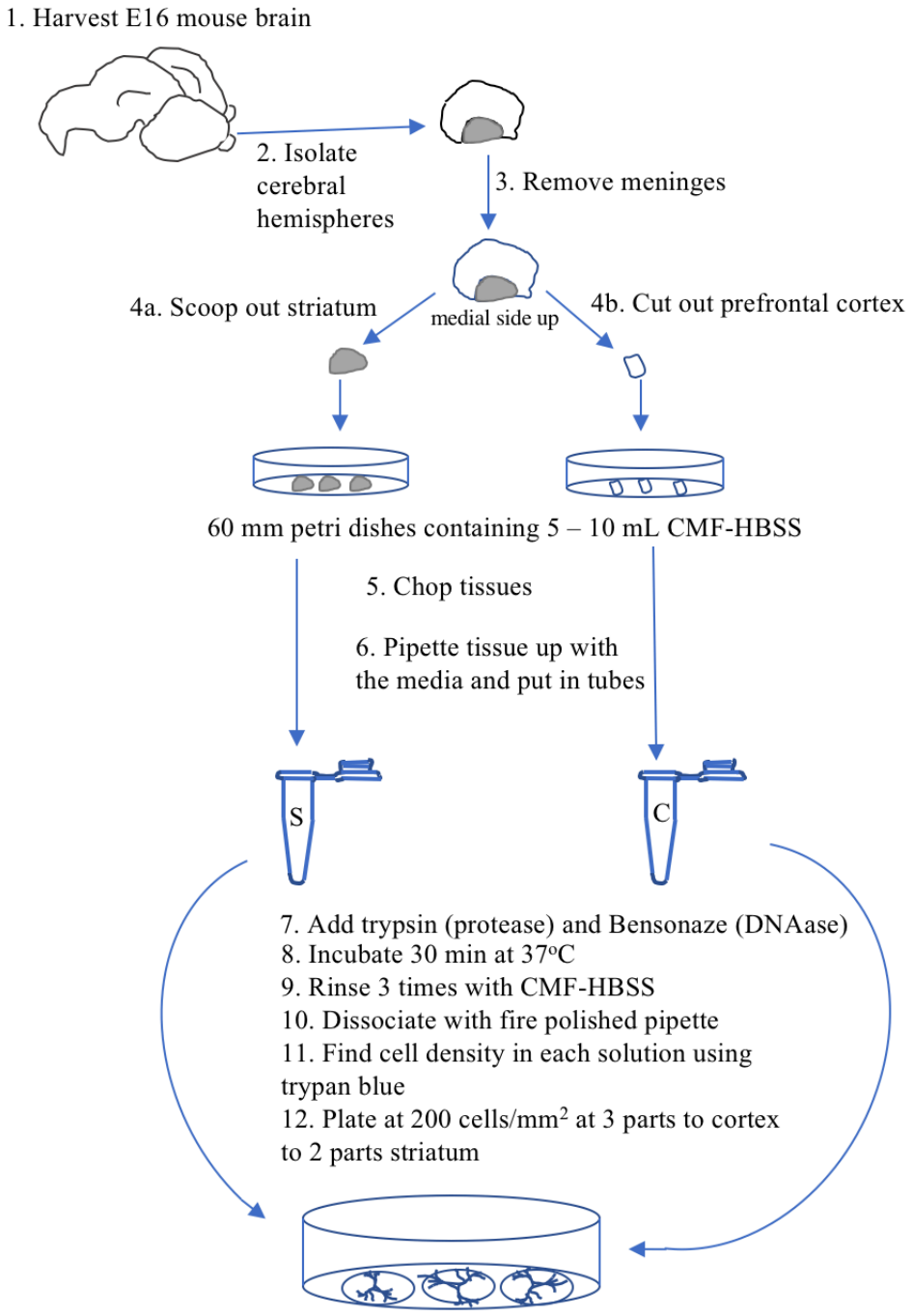
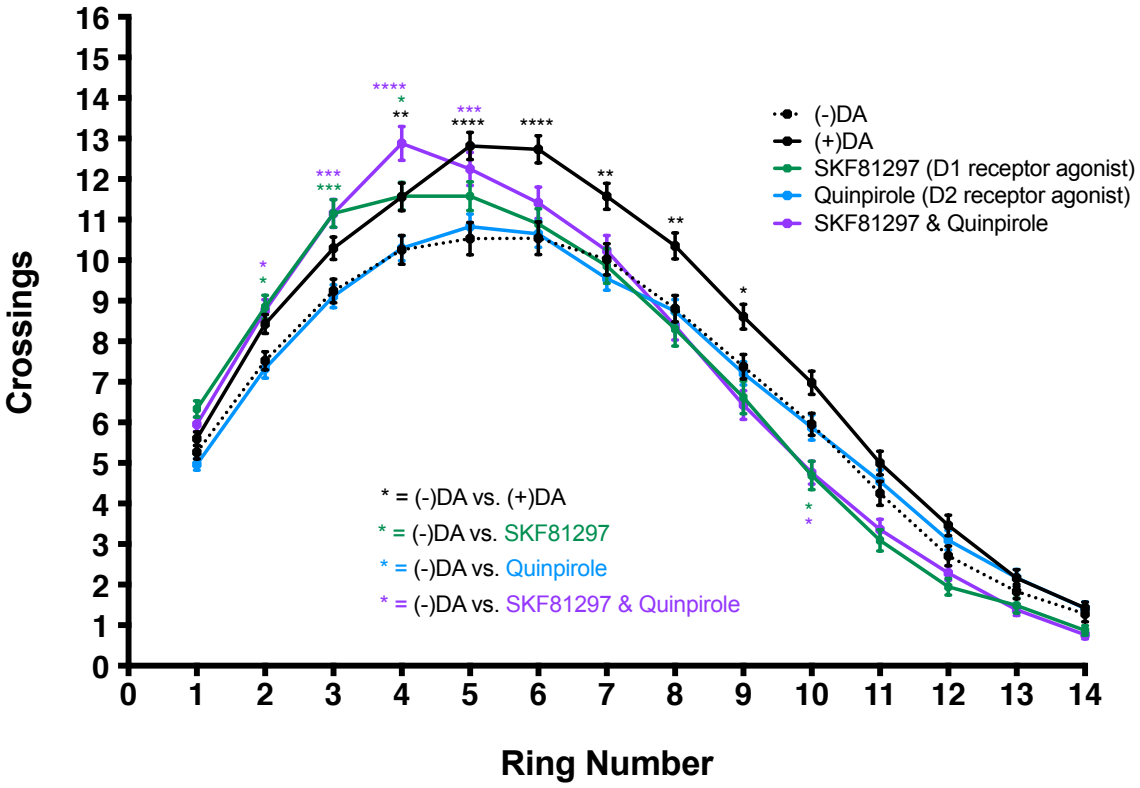
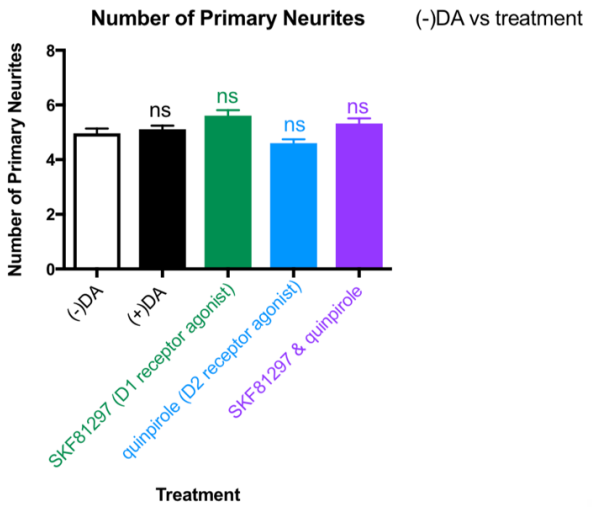


Figure 3: Schematic of the cortical striatal dissection. For additional clarification, see Methods Section 2.3: The Dissection. **Note**: Images are not to scale.

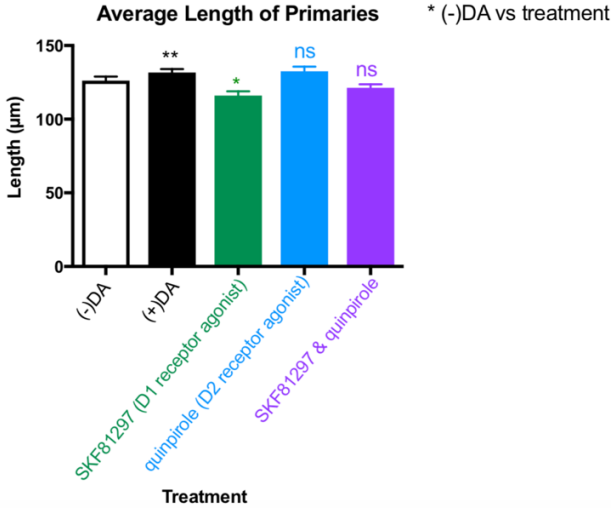
a


Crossings
Ring Number
(-)DA
(+)DA
SKF81297 (D1 receptor agonist)
Quinpirole (D2 receptor agonist)
SKF81297 & Quinpirole
* = (-)DA vs. (+)DA
* = (-)DA vs. SKF81297
* = (-)DA vs. Quinpirole
* = (-)DA vs. SKF81297 & Quinpirole

b


Number of Primary Neurites
(-)DA vs treatment
Number of Primary Neurites
ns
Treatment
(-)DA
(+)DA
SKF81297 (D1 receptor agonist)
quinpirole (D2 receptor agonist)
SKF81297 & quinpirole

c


Average Length of Primaries
* (-)DA vs treatment
Length (µm)
Treatment
(-)DA
(+)DA
SKF81297 (D1 receptor agonist)
quinpirole (D2 receptor agonist)
SKF81297 & quinpirole

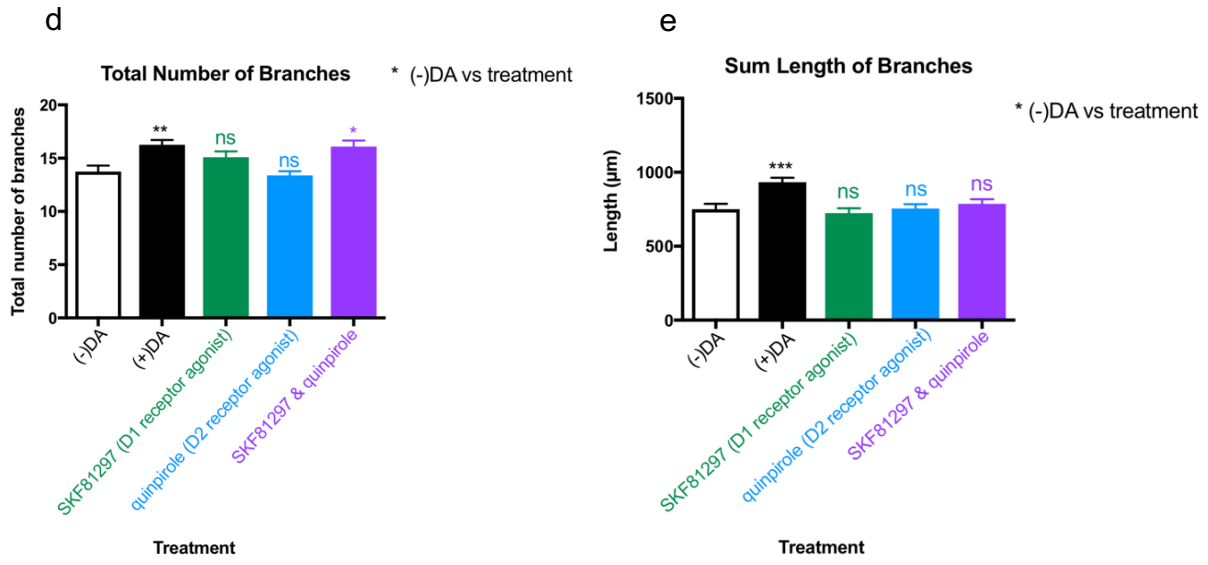


Figure 4. Dopamine receptor activation and its effect on MSN dendritic arborization. (a) Sholl analysis of MSNs treated with DA receptor agonists. One treatment was 10 µM SKF81297, a full D1 receptor agonist (green). The next treatment was 10 µM quinpirole, a D2 receptor agonist (blue). The last treatment was 10 µM of each SKF81297 and quinpirole (purple). The significant differences of each treatment were compared to (–)DA (dotted black), using a two way ANOVA, where the absence of stars means the comparison was not significantly different. For (b) through (e), (+)DA (solid black) and all treatments were compared to (–)DA, using a one way ANOVA. "ns" means no significant difference was found between the particular treatment and (–)DA. For (a) through (e), all statistical differences were annotated with stars where *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$. Data displayed as Mean ± SEM. $N = 78 – 178$ neurons for each treatment from two replicates.

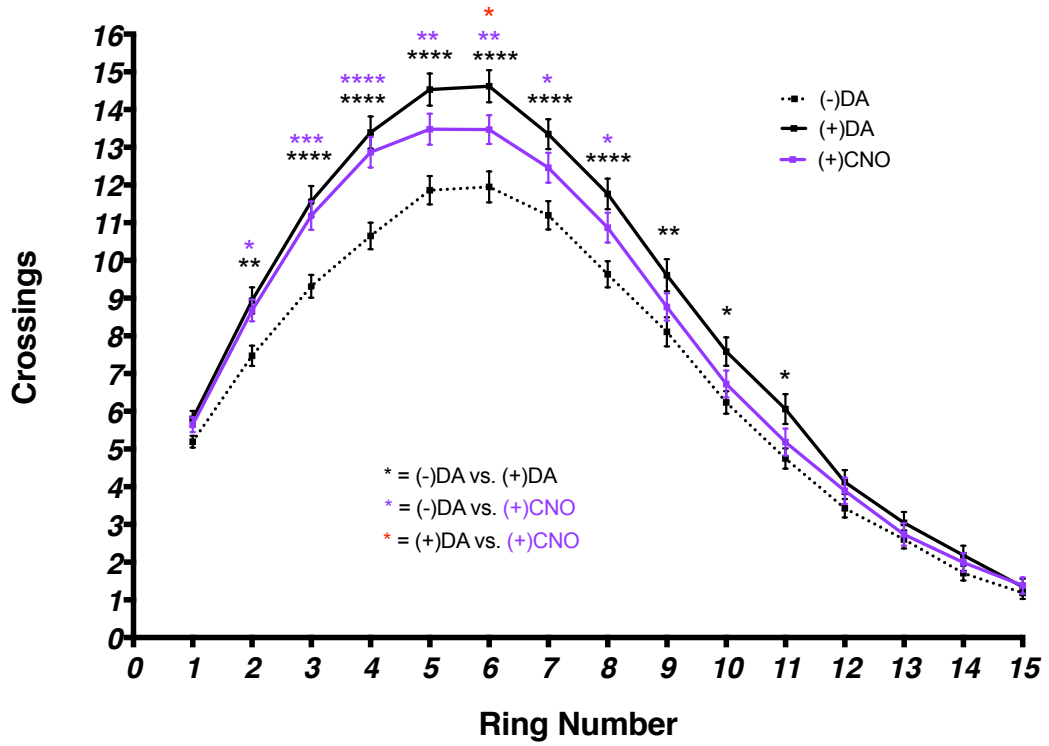


Figure 5. G_q activation and its effect on MSN dendritic arborization. Sholl analysis of MSNs treated with 12.5 nM Clozapine *N*-oxide (+)CNO. Prior to coverslip plating, the striatal tissue in the (+)CNO treatment was electroporated with the mCherry tagged G_q coupled DREADDs (Designer Receptor Exclusively Activated by Designer Drugs) plasmid. CNO was the designer drug to activate the Designer Receptor coupled to G_q. Only MSNs expressing DREADDs were traced and analyzed with Sholl to generate the data to support (+)CNO. Significant difference pairings are indicated above the x axis. Three relationships were compared for significance: (–)DA vs (+)DA (black), (–)DA vs (+)CNO (purple) and (+)DA vs (+)CNO (red) *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$, using a two way ANOVA. Data displayed as Mean ± SEM. $N = 113 – 123$ neurons for each treatment from three replicates.

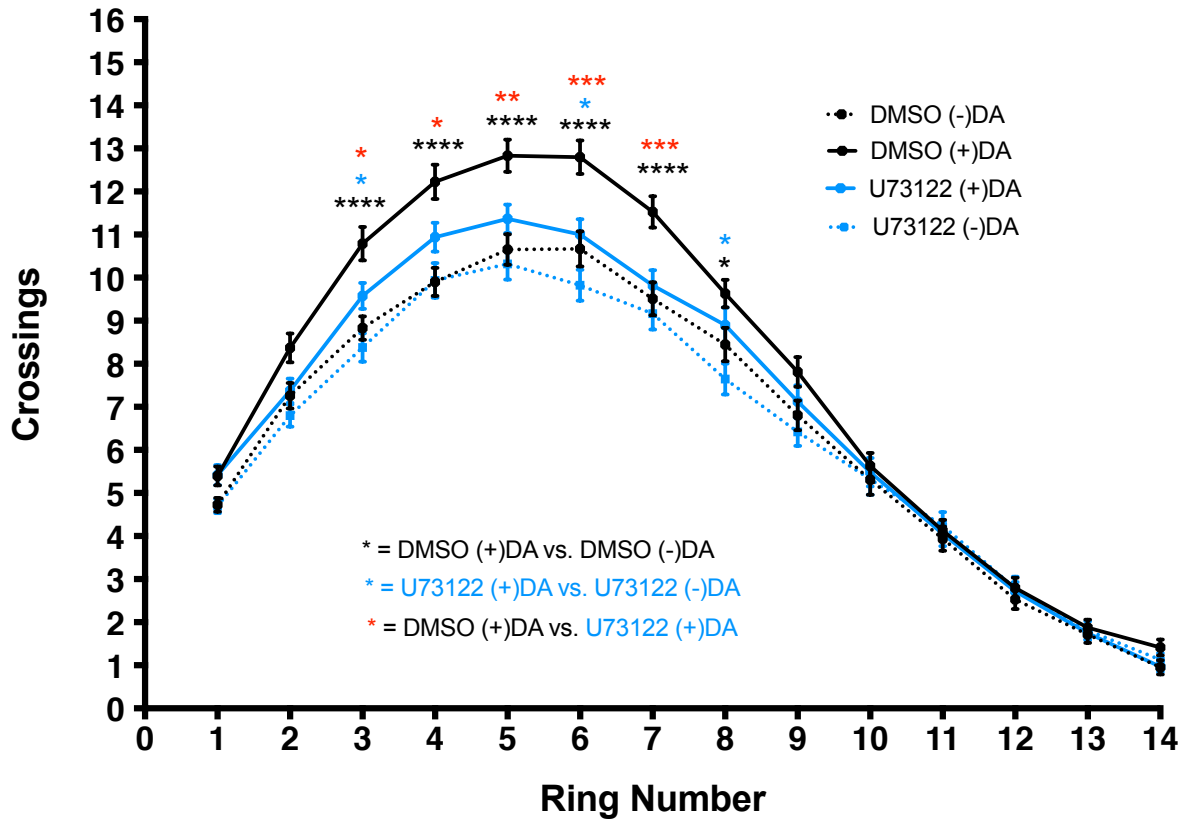


Figure 6. Phospholipase C antagonist U73122 and its effect on MSN dendritic arborization in the presence of dopamine. Sholl analysis of MSNs treated with 100 nM U73122 and (+)DA. Three different statistical analyses where compared, one between DMSO(+)DA and DMSO(–)DA (black), one between U73122(+)DA and U73122(–)DA (blue), and the last one between DMSO(+)DA and U73122(+)DA (red). U73122 is a PLC antagonist in the vehicle DMSO, so equal volume DMSO was added to (–)DA and (+)DA as a control. The three statistical comparisons used a two way ANOVA where *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$. Data displayed as Mean ± SEM. $N = 79 – 99$ neurons for each treatment.

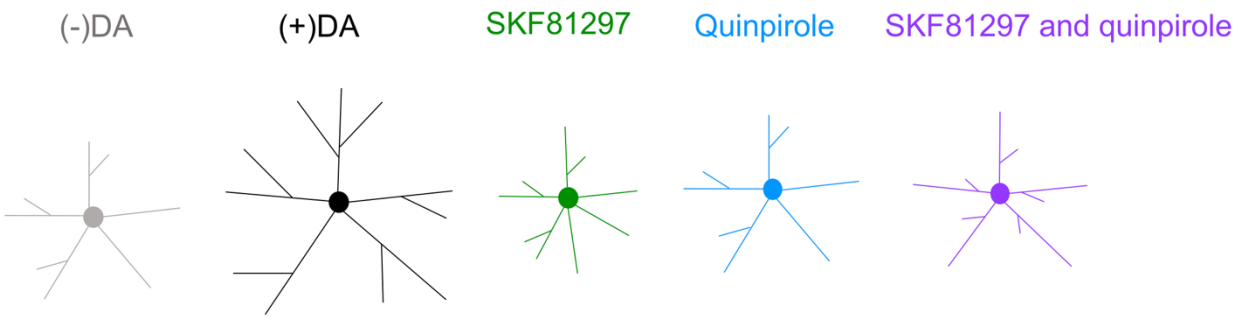


Figure 7. The prototypical neuron in each treatment from the DA receptor agonist experiment. This figure was created as an accumulation of all the data from Figure 4(a) – (e). There were no significant differences between the (–)DA MSN and the quinpirole MSN. (+)DA increased the length of the primaries and increased the number and length of branches ($p \leq 0.05$). SKF81297 decreased primary length ($p \leq 0.05$). SKF81297 & quinpirole increased the number of proximal branches. **Note**: Primary neurites are the lines extending directly from the soma. Secondaries are branches off the primaries. No tertiary or quaternary neurons are shown. These images are not to scale and the differences between treatments may be exaggerated for simplification purposes.